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CROTOXIN: STRUCTURAL STUDIES, MECHANISM OF ACTION AND CLONING OF ITS GENE

ANNUAL REPORT

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MARCH 1988

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-86-C-6061

University of Wyoming
Department of Molecular Biology
Laramie, Wyoming 82071

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS Auth
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Distribution ~~limited~~ to U.S. Gov't agencies only; Proprietary Information; March 1988.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION University of Wyoming	6b. OFFICE SYMBOL (If applicable) [redacted]
6c. ADDRESS (City, State, and ZIP Code) Dept. of Molecular Biology Laramie, WY 82071	7a. NAME OF MONITORING ORGANIZATION 7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development	8b. OFFICE SYMBOL (If applicable) SGRD-RMI-S
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, MD 21701-5012	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-86-C-6061
	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO. 3 ml-	TASK NO.	WORK UNIT ACCESSION NO.
61102A	61102BS12	AA	133

11. TITLE (Include Security Classification)

CROTOXIN: STRUCTURAL STUDIES, MECHANISM OF ACTION AND CLONING OF ITS GENE

12. PERSONAL AUTHOR(S) KAISER, IVAN I.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 1 Mar 87 TO 29 Feb 88	1988 March	

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	01		Crotoxin; Presynaptic neurotoxins; rattlesnake toxins; natural toxins; antitoxins; (IAT)
06	03		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The purpose of this project is to (1) gain greater insight into crotoxin and crotoxin homolog structure, in order to provide a better understanding of this class of rattlesnake neurotoxin; (2) develop an in vitro system for examining presynaptic neurotoxin mechanism of action; (3) clone the crotoxin gene as a first step in creating a non-toxic, but immunoreactive crotoxin analog; and (4) explore other possible non-toxic, crotoxin immunogens as potential vaccines against crotoxin and its homologs. We have completed the sequence determination of both the basic and acidic subunits of crotoxin. The acidic subunit peptides were difficult, since two of the three peptides were blocked at the amino-terminus by pyroglutamate. Four presynaptic neurotoxins and their subunits from venoms of C. d. terrificus, C. vegrandis, C. s. scutulatus, and C. v. concolor have been examined using circular dichroism, deconvolution Fourier-transform infrared, and fluorescence spectroscopy. Results indicate a large conformational change occurs upon complex formation between the acidic and basic subunits of all four toxins. (See Con't).

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ OTIC USERS	Unclassified

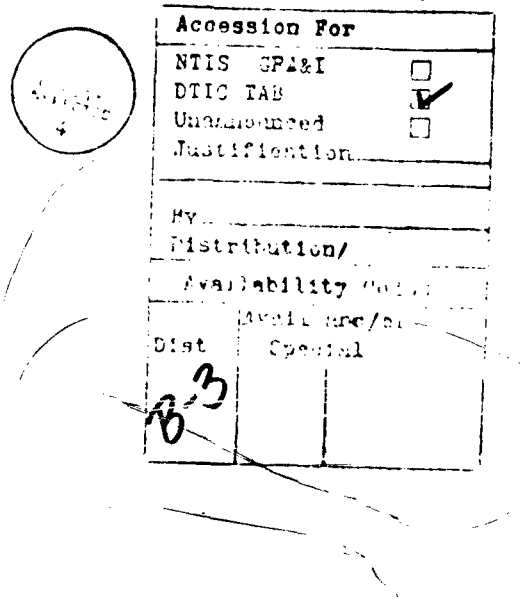
22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mary Frances Bostian	301-663-7325	SGRD-RMI-S

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

(19 Con't)

In three of the four proteins there appears to be a major loss of β-structure and a corresponding increase in non-alpha-helical structure when the complex is formed. X-ray crystallography studies should eventually provide a definitive crystalline structure for the intact crotalid presynaptic neurotoxins. Dr. Keith Ward (Naval Research Laboratories) is currently conducting such studies on the crotoxin homolog, Mojave toxin, which we purified and provided to him. Venoms from the Great Basin rattlesnake (C. v. lutosis), the Uracoan rattlesnake (C. vegrandis), and the Western diamondback rattlesnake (C. atrox), as well as Western diamondback-Mojave rattlesnake (C. s. scutulatus, Type B venom) hybrids, have been examined for crotoxin-like neurotoxins. We found such a homolog only in the Uracoan rattlesnake venom. The myotoxin fraction from C. v. concolor was also examined, as a result of earlier indications that there were sequence homologies between these smaller peptides and the basic subunit of crotoxin, as well as the existence of myotoxin isoforms. We did detect multiple isoforms, which all showed qualitatively identical myotoxic activity and behavior in double-immunodiffusion gels against antisera raised against myotoxin a. Chemical cross-linking studies on purified crotoxin are in progress, which may also provide a greater understanding of the structural requirements for toxicity. Immunological investigations with Dr. John Middlebrook (USAMRIID), have involved the preparation of polyclonal antisera raised to crotoxin, its subunits, and crotoxin homologs and a comparative study employing these antisera. We have also isolated and partially characterized four monoclonal antibodies raised to the basic subunit of crotoxin. One is a potent neutralizer of crotoxin's lethality and phospholipase A_2 activity. Anti-idiotype monoclonal antibodies have now been prepared against the neutralizing monoclonal and are being examined for their potential as crotoxin immunogens. These have been examined for possible phospholipase activity, but none was found. Initial experiments on a synaptosome system for studying presynaptic neurotoxin action in vitro have been conducted and are continuing. Additional in vitro studies with Dr. Lance Simpson (Jefferson Medical College), on the affects of crotoxin on the isolated phrenic nerve-hemidiaphragm are also underway. In collaboration with Dr. Leonard Smith (USAMRIID) we have successfully constructed a cDNA library from C. d. terrificus venom glands and a genomic library from C. d. terrificus liver DNA. Work on these libraries is in progress.



SUMMARY

The purpose of this project is to (1) gain greater insight into crotoxin and crotoxin homolog structure, in order to provide a better understanding of this class of rattlesnake neurotoxin; (2) develop an in vitro system for examining presynaptic neurotoxin mechanism of action; (3) clone the crotoxin gene as a first step in creating a non-toxic, but immunoreactive crotoxin analog; and (4) explore other possible non-toxic, crotoxin immunogens as potential vaccines against crotoxin and its homologs. We have completed the sequence determination of both the basic and acidic subunits of crotoxin. The acidic subunit peptides were difficult, since two of the three peptides were blocked at the amino-terminus by pyroglutamate. Four presynaptic neurotoxins and their subunits from venoms of C. d. terrificus, C. vegrandis, C. s. scutulatus, and C. v. concolor have been examined using circular dichroism, deconvolution Fourier-transform infrared, and fluorescence spectroscopy. Results indicate a large conformational change occurs upon complex formation between the acidic and basic subunits of all four toxins. In three of the four proteins there appears to be a major loss of ß-structure and a corresponding increase in non-alpha-helical structure when the complex is formed. X-ray crystallography studies should eventually provide a definitive crystalline structure for the intact crotalid presynaptic neurotoxins. Dr. Keith Ward (Naval Research Laboratories) is currently conducting such studies on the crotoxin homolog, Mojave toxin, which we purified and provided to him. Venoms from the Great Basin rattlesnake (C. v. lutosis), the Uracoan rattlesnake (C. vegrandis), and the Western diamondback rattlesnake (C. atrox), as well as Western diamondback-Mojave rattlesnake (C. s. scutulatus, Type B venom) hybrids, have been examined for crotoxin-like neurotoxins. We found such a homolog only in the Uracoan rattlesnake venom. The myotoxin fraction from C. v. concolor was also examined, as a result of earlier indications that there were sequence homologies between these smaller peptides and the basic subunit of crotoxin, as well as the existence of myotoxin isoforms. We did detect multiple isoforms, which all showed qualitatively identical myotoxic activity and behavior in double-immunodiffusion gels against antisera raised against myotoxin a. Chemical cross-linking studies on purified crotoxin are in progress, which may also provide a greater understanding of the structural requirements for toxicity. Immunological investigations with Dr. John Middlebrook (USAMRIID), have involved the preparation of polyclonal antisera raised to crotoxin, its subunits, and crotoxin homologs and a comparative study employing these antisera. We have also isolated and partially characterized four monoclonal antibodies raised to the basic subunit of crotoxin. One is a potent neutralizer of crotoxin's lethality and phospholipase A_2 activity. Anti-idiotype monoclonal antibodies have now been prepared against the neutralizing monoclonal and are being examined for their potential as crotoxin immunogens. These have been examined for possible phospholipase activity, but none was found. Initial experiments on a synaptosome system for studying presynaptic neurotoxin action in vitro have been conducted and are continuing. Additional in vitro studies with Dr. Lance Simpson (Jefferson Medical College), on the affects of crotoxin on the isolated phrenic nerve-hemidiaphragm are also underway. In collaboration with Dr. Leonard Smith (USAMRIID) we have successfully constructed a cDNA library from C. d. terrificus venom glands and a genomic library from C. d. terrificus liver DNA. Work on these libraries is in progress.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

BODY OF REPORT

STATEMENT OF PROBLEM

The purpose of this project is to (1) gain greater insight into crotoxin and crotoxin homolog structure, in order to provide a better understanding of this class of rattlesnake neurotoxin; (2) develop an in vitro system for examining presynaptic neurotoxin mechanism of action; (3) clone the crotoxin gene as a first step in creating a non-toxic, but immunoreactive crotoxin analog; and (4) explore other possible non-toxic, crotoxin immunogens as potential vaccines against crotoxin and its homologs.e crotoxin analog.

BACKGROUND AND APPROACH TO THE PROBLEM

The discovery of crotoxin, a potent, enzymatic neurotoxin from the venom of the South American rattlesnake (Crotalus durissus terrificus) by Slotta and Fraenkel-Conrat (1) marked the beginning of modern-day snake venom research. Crotoxin is a heterodimeric protein, consisting of a moderately toxic basic phospholipase A_2 and a acidic, non-toxic subunit composed of three small proteins (2). The acidic subunit is required for full toxicity, but has no other identified function. Evidence gradually accumulated suggesting similarity between crotoxin, Mojave toxin from Crotalus s. scutulatus, concolor toxin from Crotalus viridis concolor, and vegrandis toxin from Crotalus vegrandis, although disconcerting differences persisted (see ref. 3 and 4 for review). From our early investigations (5), we were reasonably certain that the above four toxins were similar structurally and functionally. We wanted to extend these studies and prepared rabbit antiserum against crotoxin, concolor toxin, and Mojave toxin, as well as against the acidic and basic subunits of crotoxin. These antisera were used to examine the antigenic relatedness of purified crotalid toxins and subunits by both double immunodiffusion and ELISA. We also determined the relative efficacy of antisera raised against the subunits and intact complex of crotoxin in neutralizing the toxicity of crotoxin and related toxins in mice (6). At the same time we were interested in preparing monoclonal antibodies to crotoxin. These would provide us with additional tools to probe crotoxin structure and if we were fortunate enough to generate a neutralizing monoclonal antibody, might provide for either the development of an anti-idiotype vaccine, or the identity of a neutralizing antigenic site on crotoxin (7-9).

We have also examined the venom from the Great Basin rattlesnake (C. v. lutosis), the Uracoan rattlesnake (C. vegrandis), and the Western diamondback rattlesnake (C. atrox), as well as Western diamondback-Mojave rattlesnake (C. s. scutulatus) hybrids, for crotoxin-like neurotoxins. One publication has resulted on the Uracoan study (Kaiser and Aird, 10); one on the Great Basin rattlesnake is in press (Aird, Seebart, and Kaiser; 11), and the Western diamondback results have just been submitted (Aird, Thirkhill, Seebart, and Kaiser; 12). Only in the Uracoan did we find such a crotoxin homolog.

Crotoxin has been the most extensively studied and characterized rattlesnake neurotoxins. When this work was started, the amino acid sequence of its basic subunit was largely

determined, with the exception of the Asp/Asn and Glu/Gln residues, which could not be differentiated (13). We completed the sequence of the basic subunit, and have published those results (Aird, Kaiser, Lewis, and Kruggel; 14 and 15). Two of the three acidic subunit chains, and 24-residues of the carboxyl-terminus of the third chain were also sequenced (2). Repeated attempts to sequence the blocked, amino-terminal end of the B-chain by conventional methods were unsuccessful. In 1987, we initiated a collaboration with Dr. Donald F. Hunt at the University of Virginia, whose laboratory employs tandem mass spectrometry for determining amino acid sequences in proteins. He was recently able to provide us with the amino-terminus sequence of the B-chain.

A manuscript describing this work is in preparation. Sequencing of the three peptides present in the acidic subunit, two of which are blocked by pyroglutamate, represents a significant contribution, since others have unsuccessfully attempted to sequence the peptides for the past fifteen years.

Both subunits of crotoxin have sequence homology with phospholipases A_2 even though the acidic subunit consists of three separate chains linked by disulfide bonds. However, only the basic subunit, which consists of a single polypeptide chain, actually manifests phospholipase activity. A very striking conformational change reportedly occurs in crotoxin upon complex formation (16). In contrast, when the homologous toxin from C. s. scutulatus venom (Mojave toxin) was examined, no evidence for such a conformational change was noted (Tu et al., 17). Estimates of secondary structure for these proteins conflict with x-ray crystallographic data from non-toxic, homodimeric phospholipases. In general, phospholipases A_2 appear to have approximately 50% alpha-helical structure (18), while estimates from CD and Raman studies give values of 12-18% and 70% for the C. d. terrificus and C. s. scutulatus basic subunits, respectively. Thus there is an apparent conflict in the literature regarding the existence of both conformational changes upon complex formation and the secondary structure of the subunits.

To resolve these uncertainties, we have examined the spectral properties of purified neurotoxins and their subunits from four crotalid taxa, using CD, Fourier Transform infrared (FTIR), and fluorescence spectroscopy. We find convincing evidence for a large conformational change in all cases examined, and additional evidence that the secondary structure of these toxins differs significantly from that predicted for on-toxic venom and pancreatic phospholipases A_2.

Samples of purified Mojave toxin have been provided to the laboratory of Dr. Keith Ward (Laboratory for the Structure of Matter, Naval Research Laboratory, Washington, D. C.) for crystallization studies. His laboratory has been successful in obtaining crystals suitable for x-ray diffraction studies (Norden et al., 19). Detailed x-ray structural analysis should provide valuable insight into rattlesnake presynaptic neurotoxin structure.

Previous work by Hendon and Tu (20) was designed to examine whether dissociation was essential for neurotoxicity. Dimethyl suberimidate (DMS), was used to irreversibly bind the two subunits. Their data suggest that they introduced an average of three crosslinks per complex. At least one of these must have been between subunits because recovered complex could not be dissociated in 6M urea. Sequence analyses indicate the presence of 10 lysine residues (the most likely reactant) in the basic subunit and one each in the A-chain and B-chain

of the acidic subunit (2). The DMS-crotoxin had comparable levels of phospholipase A_2 activity to that of unmodified crotoxin (21 µmol/min-mg). LD_{50} values in mice increased from 0.06 µg/g to >1.5 µg/g. Retention of phospholipase activity and loss of neurotoxicity in the cross-linked complex was interpreted to reflect "interference between the cross-linked complex and the target site." Recent results from two different groups indicate that loss of neurotoxicity may have resulted from modification of the -amino group of lysine and not necessarily cross-linking. Using chemical derivatization techniques, Rosenberg's group (21) observed that lysine or arginine group modification in basic phospholipase A_2 enzymes. frequently results in greater loss of pharmacological than of enzymatic activity. They note that modification of these basic amino acids may alter the protein's stability, distribution, or tissue binding ability. Jeng and Fraenkel-Conrat (22) also observed inactivation of neurotoxicity in crotoxin upon acetylaton of amino groups and suggested that the inactivation was due to a "discrete change in the conformation of the molecule induced by the loss of positive charges." Hendon and Tu also used DEAE-cellulose column chromatography to separate cross-linked and unreacted toxin components in phosphate-buffered urea. Their neurotoxicity loss may have resulted, in part, from carbamylations during chromatography resulting from spontaneous breakdown of urea generating cyanate which reacts with amino groups. Their unreacted controls were not cycled over urea columns. We are repeating Hendon and Tu's cross-linking experiments with the appropriate controls,and have examined four different chemical cross-linkers, plus one lysine-specific monofunctional reagent in the crotoxin modification studies. Our efforts are now directed toward the most promising of these, 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide. It appears to efficiently cross-link the subunits of crotoxin. We have developed an isolation procedure for the cross-linked crotoxin on Sephacryl S300 in 6M urea and are presently characterizing this material. The chemical modification phase of the work should be complete by the end of the current contract period.

We have also examined the myotoxin fraction from C. v. concolor. Goncalves (23) reported the presence of a highly basic polypeptide from the venom of C. d. terrificus, which he named crotamine. Since that time, crotamine-like proteins have been reported in the venoms of a number of different crotalids. Although the exact biological mode of action of these myotoxins is not known, it is clear that their first microscopically observable effect is on muscle cells, causing vacuolation (Ownby et al., 24). The purpose of the work by us was to determine whether the minor structural differences between isotoxins isolated from one batch of venom resulted in any biological and immunological differences. This has implications to our work on crotoxin, because of the crotoxin and crotoxin-homolog isoforms recently identified by us and others (10, 25). In addition, earlier sequence comparisons suggested that there were some sequence homologies between these smaller peptides and the basic subunit of crotoxin.

By 1986, we thought we would have available an in vitro system of synaptosomes and/or tissue culture cells with well defined responses to crotalid neurotoxins. This was being overly optimistic. It is 1983 and an optimum system is still not available. We have conducted preliminary experiments using guinea pig brain synaptosomes and ^{3}H-choline uptake and release measurements to monitor responses to crotalid neurotoxins. Existing assays and procedures have proven to be more extensive and involved than originally anticipated. New

methodology, described in the renewal application will be employed to further evaluate synaptosomes as a system for studying presynaptic neurotoxin action in vitro.

We have also initiated a collaboration with Dr. Lance Simpson (Jefferson Medical College), who has examined crotoxin for its neuromuscular blocking properties on the isolated phrenic nerve-hemidiaphragm preparation from the mouse. He finds the toxin produces concentration-dependent paralysis of transmission with a high temperature dependence. Our crotoxin-neutralizing monoclonal antibody at equimolar concentration, abolishes the toxicity if premixed prior to addition to tissues. If antibody is added to tissues after the toxin has become bound, it no longer affords protection. One of our non-neutralizing monoclonal antibodies at equimolar concentrations to crotoxin, does not delay onset of neuromuscular blockage.

Little is known about the biosynthesis of crotoxin or for that matter any venom proteins found in reptiles. This cloning work should permit us to answer fundamental questions concerning crotoxin and related crotalid neurotoxins. It will also provide a cloned crotoxin gene that will permit manipulation and selective alteration. In collaboration with Dr. Leonard Smith (USAMRIID), we located five C. d. terrificus snakes and have now successfully constructed (1) a cDNA library in a lambda vector using poly (A+) RNA from C. d. terrificus venom glands and (2) a genomic library in lambda from C. d. terrificus liver DNA. In addition, we have identified a 20kb fragment from the genomic DNA of C. atrox that hybridizes with one of our synthetic probes for the basic subunit of crotoxin.

RESULTS

I. MONOCLONAL ANTIBODIES. Four different monoclonal antibodies, typed as IgG_1 subclass, were raised against the basic subunit of this toxin. One was a potent neutralizing antibody of intact crotoxin, which could neutralize ≈1.6 moles of purified crotoxin per mole of antibody. The monoclonal antibody enhanced the neutralizing ability of commercial polyvalent crotalid antivenin against the lethality of crude C. d. terrificus venom four fold. Using an enzyme-linked immunosorbent assay, we tested various proteins for competitive inhibition of binding of biotinylated-crotoxin to plates coated with the four individual monoclonal antibodies. Concolor toxin, vegrandis toxin, intact crotoxin, Mojave toxin, and the basic subunit of crotoxin showed increasing effectiveness as displacers of crotoxin from the neutralizing monoclonal antibody. Results are summarized in Table 1. **None** of the monoclonal antibodies reacted with purified phospholipase A_2s from Crotalus atrox or Crotalus adamanteus, nor any of the components present in the crude venoms from four different elapids known to contain presynaptic neurotoxins, which show some sequence identity to crotoxin. Experimental details may be found in ref. 8. Work is in progress on the affects that the four different monoclonal antibodies have on the phospholipase activity of crotoxin.

II. MOUSE ANTI-IDIOTYPE MONOCLONAL ANTIBODIES RAISED AGAINST THE CROTOXIN NEUTRALIZING MONOCLONAL ANTIBODY. Nine anti-idiotype ascites fluids raised against the crotoxin neutralizing monoclonal antibody (line 1), were received from Dr. John L. Middlebrook at USAMRIID. These were stored at -80°C until used.

Samples were thawed, spun in a microfuge, and supernatants aspirated. Total protein assays (BioRad) on the supernatants and gave values ranging from 26.6 to 43.3 mg/ml. Supernatants were then run on SDS-PAGE on 7.5% gels for 6 hours at 20 mA. Results are shown in Figure 1. About 20 µg of protein was run per slot. Phospholipase assays (Aird and Kaiser, 5) were run directly on each ascites fluid supernatant, with no evidence of any activity. An attempt to purify each monoclonal antibody from its ascites fluid was then carried out by a combination of ammonium sulfate precipitation and Protein A affinity chromatography (8). Yields were low in all cases, as expected, since the gels on the crude ascites did not show significant quantities of antibodies in the ascites fluids (see Figure 1). Nevertheless, we were able to isolate small amounts of anti-idiotype monoclonal antibodies 2, 4, 6, and 9. These were examined by SDS-PAGE (Figure 2), and assayed for phospholipase activity. No phospholipase activity was found in any of the four purified anti-idiotype monoclonal antibodies examined.

III. CROTOXIN HOMOLOGS. (A) CROTALUS VEGRANDIS. A major protein toxin from the venom of Crotalus vegrandis was examined by gel filtration, anion-exchange chromatography, and SDS polyacrylamide gel electrophoresis. The toxin was separated into several isoforms by ion-exchange chromatography and spontaneously dissociated into free acidic and basic subunits, mimicking the behavior of crotoxin. Rabbit antisera raised against crotoxin reacted strongly in enzyme-linked immunosorbent assays with the intact C. vegrandis toxin isoforms and their basic subunits, and formed precipitin lines of identity with intact crotoxin in double immunodiffusion gels. These results indicate that vegrandis toxin is strongly homologous with crotoxin from the venom of Crotalus durissus terrificus. Experimental details may be found in the year 1 report or the recently published paper (10).

(B) CROTALUS VIRIDIS LUTOSIS. No additional work has been carried out on this species since the year 1 report. As noted there, we found no evidence for the presence of a crotoxin homolog in the venom (11).

(C) CROTALUS ATROX and CROTALUS ATROX-CROTALUS SCUTULATUS SCUTULATUS HYBRIDS. We examined the venom composition and morphology of a male C. atrox, a female C. s. scutulatus, their F_1 hybrid offspring, and a half sibling C. s. scutulatus. F_1 hybrids show morphological and venom characteristics of both parental species. F_2 hybrids show morphological characteristics of both C. atrox and C. scutulatus. Type B C. scutulatus venom (that lacking Mojave toxin) is shown to be very similar to that of C. atrox and qualitatively very different from Type A C. scutulatus venom (that containing Mojave toxin). We found no evidence of Mojave toxin in any of the venoms examined. An earlier report of the occurrence of Mojave toxin in the venom of C. atrox by Minton and Weinstein (26) has been critically evaluated. We conclude that they probably mis-identified non-toxic phospholipase A_2 for the basic subunit of Mojave toxin. A manuscript describing this work has been submitted for publication (12).

IV. PROTEIN SEQUENCING. CROTOXIN. (A) BASIC SUBUNIT. Determination of the primary

sequence of the basic subunit has been completed. See year 1 report and references (14,15).

(B) ACIDIC SUBUNIT. A partial sequence was reported in the year 1 report and reference (2). Various attempts to sequence the blocked, amino-terminal end of the acidic subunit B-chain by conventional methods were unsuccessful. In 1987, we initiated a collaboration with Dr. Donald F. Hunt at the University of Virginia, whose laboratory employs tandem mass spectrometry for determining amino acid sequences in proteins. We provided him with purified B-chain of crotoxin's acidic subunit. In mid-December, 1987, he and his associates were able to report to us the amino-terminus sequence of the B-chain, which is shown below.

```
          70                        80
pQ-E-D-G-E-I-V-C-G-E-D-D-D-P-C-...
```

From previous work, we expected ten residues to precede the Asp at position 79. Instead we found twelve. It is unclear why pyroglutamate aminopeptidase was unable to remove the amino-terminal group as it did from C-chain. This end is extremely acidic, with glutamate and aspartate representing the next two amino acids in from the N-terminus, with seven of the first thirteen amino acids being acidic.

Sequencing of the three peptides present in the acidic subunit, two of which are blocked by pyroglutamate, represents a significant contribution. Others have attempted to sequence the acidic subunit for the past fifteen years, but were unsuccessful.

V. MOJAVE TOXIN X-RAY STUDIES. Purified Mojave toxin was recrystallized from an initially hot pyridine-acetic acid solution to yield a few diffraction quality single crystals. This crystalline form is characterized by a unit-cell with parameter values a=38.6, b=69.9, c=77.6 Å, and alpha=beta=gamma=90 degrees. Its diffraction symmetry, any anomalous scattering ignored, is Pmmm for which the unique space group assignment P(2/1)(2/1)(2/1) is required to account for the systematic extinctions observed. A complete x-ray diffraction record was measured using a Xentronics area detector system on a rotating-anode x-ray generator. Although the crystals diffract to a resolution of better than 2.0 Å (minimum interplanar spacing), the data set has an effective resolution of 2.1 Å at which over 11,000 reflection intensities were measured with an internal agreement of ca. 7%. The results from the area detector system were corroborated by photographic studies at the Naval Research Laboratory's synchrotron beam line at Brookhaven.

At present all attempts have failed to produce Mojave toxin crystals tagged with heavy atoms or groups for phase problem solution by isomorphous replacement methods. Initial structure solution has come from molecular replacement methods. Initial structure solution has come from molecular replacement techniques using a 'trimmed' phospholipase monomer as the model. A clear rotation-translation solution was obtained for the phospholipase modeled basic subunit. After extensive calculation, the acidic subunit also was placed in the unit cell, though with less definition. The structure model has been started through an interactive plan of stereochemically restrained least-squares refinement and subsequent model rebuilding. The computer-graphic displays of the current structure model and electron density maps suggest the basic subunit is better defined than the three-segment acidic subunit. This is in agreement

with the pattern of the refinement results. The current standard crystallographic R value for data at 3.0 Å resolution is just under 0.40. This is consistent with a good initial model that needs a lot of refinement and rebuilding. These initial results suggest, as do the sequence and primary structure, the basic subunit will closely resemble a canonical phospholipase in three-dimensional structure. The chaperone unit, on the other hand, even if recognizable as similar to such a canonical structure, will be significantly different.

Bieber's laboratory. (27), recently reported the sequence of the A- and C-chains of the acidic subunit of Mojave toxin from the venom of C. s. scutulatus. They found the sequences of these two chains to be exactly homologous to the sequences of the A- and C-chains of the acidic subunit of crotoxin that we reported (2), except that they differ in chain length. Relative to crotoxin, the C-chain is missing the N-terminal pyroglutamate and phenylalanine. The A-chain is lacking the two residues at the N-terminus and three residues at the C-terminus, respectively. They reported B-chain to be blocked by an unknown agent in the above reference, but have subsequently determined its sequence in collaboration with Donald Hunt's laboratory.

Bieber is not sequencing the basic subunit of Mojave toxin (personal communication, 1987). Because Keith Ward is approaching the time when he will soon need sequence information on both subunits from Mojave toxin to refine the x-ray structure analysis on Mojave toxin, we have initiated purification of additional basic subunit for sequence studies. Based on our immunological and physical studies, we feel there will be great similarity between the basic subunit of Mojave toxin and crotoxin. Thus, much of the experience gained with crotoxin should be directly applicable to the basic subunit of Mojave toxin and greatly facilitate this sequencing effort. We would like to delay the actual sequencing effort until later this year or even next year if at all possible, but it will be somewhat dependent upon the progress that Ward's laboratory makes on structural refinement and his need for the data.

VI. SPECTRAL MEASUREMENTS. Crotalid presynaptic neurotoxins are heterodimeric proteins containing an acidic and basic subunit (5). Both subunits have sequence homology with phospholipases A_2 even though the acidic subunit consists of three separate chains linked by disulfide bonds. However, only the basic subunit, which consists of a single polypeptide chain, actually manifests phospholipase activity. A very striking conformational change reportedly occurs in crotoxin upon complex formation. Marked changes in both fluorescence and circular dichroism (CD) spectra are reported to appear when the subunits interact, suggesting a large change in the structure of either one or both subunits (Hanley, 16). In contrast, when the homologous toxin from C. s. scutulatus venom (Mojave toxin) was examined, no evidence for such a conformational change was noted (Tu et al., 17). Estimates of secondary structure for these proteins conflict with x-ray crystallographic data from non-toxic, homodimeric phospholipases. In general, phospholipases A_2 appear to have approximately 50% alpha-helical structure (18), while estimates from CD and Raman studies give values of 12-18% and 70% for the C. d. terrificus (16) and C. s. scutulatus (17) basic subunits, respectively. Thus there is an apparent conflict in the literature regarding the existence of both conformational changes upon complex formation and the secondary structure of the subunits.

In order to resolve these uncertainties, we have examined the spectral properties of four

presynaptic neurotoxins from the venoms of C. d. terrificus, C. vegrandis, C. s. scutulatus and C. v. concolor. These were examined by circular dichroism (CD), deconvolution Fourier-transform infrared (FTIR), and fluorescence spectroscopy. CD and FTIR spectra indicate that the basic subunits possess 16-22% alpha-helix, as compared to 40-50% for phospholipases generally. These spectra also demonstrate that the intact toxins exhibit substantially less ß-sheet than is predicted from spectra of free acidic and basic subunits. In all but the toxin from C. vegrandis, the difference between observed and predicated values is accounted for primarily by an increase in "remainder" structure rather than alpha-helix. The implication of these findings is that a major conformational change occurs upon subunit dissociation, presumably as an increase in ß-sheet in the basic subunit, which is the toxic phospholipase moiety. Fluorescence spectra of free subunits and intact toxins show that the basic subunits are considerably more fluorescent than the acidic subunits, consistent with known tryptophan contents. Three of the four intact toxins exhibit significant (50-80%) fluorescence quenching as compared to spectra predicted by summation of free acidic and basic subunit spectra. The intact toxin from C. vegrandis actually exhibits a 30% increase in fluorescence as compared with the predicted spectrum. These data are interpreted as arguing in favor of a loss of regular secondary structure in one or both subunits during complex formation.

A paper and abstract describing these results has recently been submitted for publication (28, 29) and may be consulted for additional details.

VII. MYOTOXINS. Myotoxins from C. v. concolor were isolated by gel filtration. The crude myotoxin peak was subfractionated into either two or four subfractions by cation exchange FPLC, depending upon the source of the venom. When injected at 2 µg/g, crude concolor myotoxin caused vacuolation of mouse muscle cells typical of myotoxin a from C. v. viridis and crotamine from C. d. terrificus. All four subfractions showed qualitatively identical myotoxic activity. In double immunodiffusion studies, myotoxin a antiserum produced lines of identity when reacted with myotoxin a, crude concolor myotoxin, and the four concolor subfractions. A second batch of material showed two major components when subfractionated by cation exchange FPLC. The more basic of these two components displayed approximately twice the intravenous lethality of the more acidic component. The LD_{50} for the basic component lies between 0.625 µg/g and 0.75 µg/g while that of the acidic component falls between 1.00 µg/g and 1.25 µg/g. For additional details, see reference by Ownby, Aird, and Kaiser (30).

VIII. IN VITRO ASSAYS TO EXAMINE NEUROTOXIN MECHANISMS. We have conducted preliminary experiments using guinea pig brain synaptosomes and ^{3}H-choline uptake and release measurements to monitor responses to crotalid neurotoxins. Existing assays and procedures have proved to be more extensive and involved than originally anticipated. New methodology, involving bioluminescence (31) to monitor the continuous detection of acetylcholine release from synaptosomes, or other tissues, has been examined. Preliminary studies using model systems and a luminometer in another laboratory, are encouraging. We intend to pursue the application of bioluminescence to acetylcholine detection during the next year.

We have collaborated with Dr. Lance Simpson (Jefferson Medical College) on the affects of crotoxin and its neuromuscular blocking properties on the isolated phrenic nerve-hemidiaphragm preparation from the mouse. The same system has also been used to determine the effectiveness of our neutralizing monoclonal and one non-neutralizing monoclonal antibody, on inhibiting crotoxin's neuromuscular effects.The major findings can be summarized as follows.

1. Crotoxin produces concentration-dependent paralysis of transmission. The potency of the material appears to be at least 5-fold greater than that of preparations previously described in the literature.

2. Neuromuscular blockade is temperature dependent.

3. Drugs that are known to antagonize the actions of certain internalized substances have been tested a putative antagonists of crotoxin. However, ammonium chloride (1-8 mM) and methylamine hydrochloride (1-15 mM) offer no protective effect.

4. The neutralizing monoclonal antibody has been tested on the neuromuscular junction. The antibody itself does not alter transmission, but it is an effective antagonist of crotoxin. The monoclonal antibody, at approximately equimolar concentrations, abolishes the toxicity of crotoxin. This result is obtained only when antigen and antibody are mixed prior to addition to tissues. If antibody is added to tissues after the toxin has become bound, it no longer affords any protection.

5. One non-neutralizing antibody has been tested. At equimolar concentrations with crotoxin, it does not delay onset of neuromuscular blockade. When tested at a large molar excess (10-fold), the antibody may have weak neutralizing activity.

6. The monoclonal antibodies have been tested against one heterologous phospholipase A_2 neurotoxin, ß-bungarotoxin. No neutralizing activity was observed.

IX. CLONING. (A) Cloning of toxin genes from Crotalus durissus terrificus. In the past year we were able to locate and procure five Crotalus durissus terrificus from Herptofauna, Fort Myers, Florida. These snakes were milked and sacrificed three days later. Venom glands and liver were surgically removed and quickly frozen in liquid nitrogen. They were shipped on dry ice to to Dr. Leonard Smith, Pathology Division, U.S.A.M.R.I.I.D., at Ft. Detrick. Total RNA (375µg) was extracted from 1 gram of gland tissue, which yielded 5µg of poly (A+) RNA. A cDNA library was constructed in a lambda vector using 2µg of the poly (A+) RNA. The library yielded a titer of 3.4×10^6 pfu/ml. Two probes were synthesized (one for acidic and one for the basic subunit of crotoxin) and used to screen 500,000 primary clones. Fifteen acidic subunit positives were picked on the first round of screening. The second round screening of these fifteen yielded 8 strong positives. These eight are being subcloned into a plasmid vector and mapped. Some of these will then be sequenced. The synthetic probe designed for the basic subunit was used to screen 500,000 primary clones. No positives resulted from this screening. Our strategy is to use the acidic subunit cDNA as a probe to screen for the basic subunit.

A genomic library was constructed from DNA isolated from the liver of C. d. terrificus. The amplified titer in E. coli K802 was 4.2×10^9 pfu/ml. We screened 400,000 clones and on the

second round screening picked 46 strong positives. One of these, having an insert of 9kb, has been grown up on a preparative scale. This is now being mapped and subcloned. We are presently screening additional clones for the acidic and basic subunit of crotoxin.

The cDNA and genomic libraries from C. d. terrificus promise to be useful to other workers. For example, Dr. Catherine Alcaide, working in Dr. Smith's laboratory, has used a myotoxin cDNA which she cloned and sequenced earlier to probe and isolate the crotamine cDNA. Eight hundred positives were detected in a first round screening of 400,000 clones. Ten were picked and eight strong positives resulted from the second round screening. These eight are presently being subcloned, mapped, and some will be sequenced.
In addition, Dr. Smith in collaboration with Dr. Lance Simpson, will be isolating the gene for gyroxin from the cDNA and genomic libraries.

(B) Cloning of the Phospholipase A_2 gene from the Western Diamondback rattlesnake (Crotalus atrox).

There is about 50 percent sequence identity between C. atrox phospholipase A_2 (122 amino acids) and the basic subunit of crotoxin, although the C. atrox phospholipase A_2 possess no neurotoxicity. It may eventually be possible to combine different gene segments of the basic subunit of crotoxin and the non-toxic C. atrox phospholipase A_2 to form a "hybrid" phospholipase and identify toxic domains in the presynaptic neurotoxin. With this in mind and also to gain some experience in manipulating Crotalus DNA, we initiated studies to clone the phospholipase A_2 gene from C. atrox.

To date, we have successfully (1) isolated C. atrox genomic DNA, (2) probed total genomic DNA in a dot-blot assay, and (3) carried out southern blots of agarose gels of genomic DNA restriction enzyme digests. Our probe consisted of a redundant synthetic 17-mer (53-76% GC; 64 degeneracy) complementary to the C. atrox phospholipase antisense strand, corresponding to an internal amino sequence from Tyr-27 to Gly-32. This sequence is identical to the corresponding sequence in the basic subunit of crotoxin. Complete BamHI digests of genomic DNA release a 20kb fragment containing the phospholipase gene. This fragment will be eluted from the gel, ligated and packaged into EMBL3, a lambda replacement vector. Future goals include restriction mapping, sequencing, and finally orientation of the gene in the proper position in an expression vector to attempt initial translation in a bacterial system. Because of the presence of seven disulfides in the phospholipase, it seems unlikely that a fully processed, mature and active phospholipase can be recovered from a bacterial system.

DISCUSSION

Of the four monoclonal antibodies raised against the basic subunit of crotoxin, one had excellent neutralization potential against purified intact crotoxin. On a molar basis, one mole of purified monoclonal antibody effectively neutralized ≈1.6 moles of crotoxin. The neutralizing monoclonal antibody was effective in neutralizing Mojave toxin present in crude venom and appeared less effective in neutralizing purified concolor toxin. This same pattern was seen in competition experiments with biotinylated-crotoxin, where intact crotoxin, Mojave toxin, and

vegrandis toxin showed greater competition with the modified toxin than did concolor toxin. Non-toxic phospholipase A_2 from C. atrox showed no affinity for any of the four monoclonal antibodies nor did any of the components from four elapid venoms, O. s. scutellatus, B. multicinctus, Pseudonaja textilis, and N. scutatus.

Despite extensive crotoxin microheterogeneity (12), the antigenic sites recognized by monoclonal antibodies 1, 2, and 5 appear conserved, since all showed similar reactivity with six different lots of crude C. d. terrificus venom and intact crotoxins from three different suppliers. With monoclonal antibodies 2, 5, and 11, we observed that the acidic subunit was a good competitor with biotinylated-crotoxin. These findings indicate that the epitopes recognized by the monoclonal antibodies were common to both the acidic and basic subunits. In sequence studies, we observed 55% sequence identity between the subunits of crotoxin over the 76 known residues of the acidic subunit (2,15). Yet, the non-toxic phospholipase A_2 from C. atrox, which shows about 50% sequence identity with the basic subunit, gave no cross-reaction with any of the four monoclonal antibodies. A detailed sequence comparison of these proteins and others may provide clues as to the sites recognized by the antibodies.

The phospholipase A_2 active site region from a variety of different snake venoms, including C. atrox, shows absolute conservation of residues from amino acids 41-53 (see14). Position numbers correspond to sequence alignments in that reference. In addition, a "catalytic network" of four residues, including His-47, has been identified as essential in the catalytic activity of phospholipase A_2 from C. atrox. These residues are also conserved in all phospholipases. We observed no cross-reaction between our monoclonal antibodies and phospholipase A_2 from C. atrox, suggesting that neither residues 41-53 nor the other three residues that make up the catalytic network (Tyr-51, Tyr-65, and Asp-90) are a significant part of the recognition site for any of the four monoclonal antibodies. If the catalytic region is the epitope or antigenic site, it is not available in C. atrox phospholipase A_2 for reaction.

It is clear that the availability of neutralizing and non-neutralizing monoclonal antibodies raised to crotoxin provide an additional approach for examining the structural basis of its toxicity. If the antigenic region(s) of crotoxin which bind to neutralizing antibodies are linear peptides, it may be possible to chemically synthesize these regions, couple them to carrier molecules, and use them for vaccination purposes.

Minton and Weinstein (26) reported the occurrence of Mojave toxin in small amounts in C. atrox venom. This claim was based on ELISA, western blots and SDS-PAGE. We are hesitant to accept that conclusion based on the data they present. We found that polyclonal antibodies raised in rabbits against crotoxin react weakly with non-toxic, homodimeric phospholipase A_2 from C. atrox. Thus, it is imperative that controls be run to demonstrate that the assay system employed cannot detect related, but different proteins. In SDS-PAGE the basic subunit of crotalid presynaptic neurotoxins and monomers of non-toxic phospholipases A_2 frequently co-migrate exactly (unpublished observations). Accordingly, it is not sufficient to show the presence of a band with a molecular weight of 14 to 15 kD. Western blots also may not be well suited to the demonstration of crotalid presynaptic neurotoxins. Our experience with this method indicates that the acidic subunit does not blot well, although the reason for this is not entirely clear. Again, as with ELISA, the use of polyclonal antibodies also detected non-toxic

phospholipases. Our ELISA data, using monoclonal antibodies against the basic subunit of crotoxin, show all C. atrox samples examined to date, including both pooled commercial samples to be negative for Mojave toxin. If one wishes to demonstrate the presence of a crotalid presynaptic neurotoxin, it is essential to show that both the acidic and basic subunits are present. Since the acidic subunit stains weakly with an acid stain such as Coomassie Brilliant Blue, it is advisable to use a sensitive, differential color silver stain such as Gelcode.

Based on secondary structure estimates using both CD and FTIR, the conformations of crotoxin and concolor toxin appear quite similar. The acidic subunits from C. vegrandis and C. s. scutulatus contain significantly more ß-structure than the corresponding subunits of C. d. terrificus and C. v. concolor.

Crystal structures of several phospholipases A_2 suggest that in general, phospholipases contain about 50% alpha-helix. Estimates of alpha-helical content based on CD from a wide variety of these enzymes support a mean estimate of 40% helix (18). Studies of basic subunits from C. d. terrificus and C. s. scutulatus neurotoxins using CD and Raman spectroscopy have given values of 12-18% (16) and 67% (17), respectively. Our estimates of alpha-helix range from 16-22% (Table 2) for the four basic subunits, agreeing with those of Hanley (16) for crotoxin. Similarly, our estimate of 35-51% ß-structure for the basic subunits is much greater than the previous Mojave toxin estimate (16%, Tu et al., 17), but in agreement with the CD-based estimates of 37-40% for C. d. terrificus (16). The basic subunits of the four crotalid neurotoxins do not manifest the high alpha-helix content generally proposed for phospholipases A_2 from crystal structures (18).

Using the recently determined sequence of the basic subunit of C. d. terrificus (14), we have also performed secondary structure analyses using the methods of Chou and Fasman (32) and Burgess (33). Both algorithms predict significantly more ß-structure than alpha-helix in the basic subunit, in agreement with experimental results. This suggests a major structural difference between the crotalid neurotoxin basic subunits and non-toxic crotalid venom phospholipases A_2. This could be related to the presence of structural elements necessary for subunit interactions or be due to intrinsic differences between other phospholipases A_2 and crotalid neurotoxin basic subunits.

The most striking finding in this work is the CD and fluorescence evidence for a large conformational change upon complex formation between the acidic and basic subunits of all four proteins. Evidence for such a change was previously reported (16) for C. d. terrificus, but similar studies (17) with C. s. scutulatus suggested no such alterations in structure. The reason for the discrepancy between our results and those of Tu et al. (17) is unknown, but the large observed spectral changes upon complex formation in all four crotalid neurotoxins examined suggests that this is a general feature of these proteins. In three of the four proteins, there appears to be a major loss of ß-structure and a corresponding increase in non-alpha-helical structure when the complex is formed. Our interpretation of these changes differs from that of Hanley, who reported an increase in ß-structure upon complex formation.

The apparent conformational change is also accompanied by a large quenching of intrinsic tryptophan fluorescence of the subunits. If we assume that the change in structure is due to the appearance of relatively disordered structure (e.g. coil as opposed to alpha-helix or ß-sheet),

this strongly argues that formation of the intact toxin is accompanied by a loss of regular secondary structure within one or both subunits, and that the creation of the subunit interface itself (and consequent interfacial structure) is not responsible for the observed spectroscopic changes. It is interesting to note that the fluorescence maxima of the tryptophans are not markedly shifted during complex formation. This suggests that they remain in relatively polar environments but become quenched by static processes in their new environment. This could be explained by transfer from a relatively solvent-exposed environment in the free subunits, to polar subunit interfaces. This idea is supported by the polar nature of the subunit interface of a dimeric phospholipase A_2 from C. atrox (34) and implies that the large changes in tryptophan fluorescence may not be due directly to the large conformational changes suggested by the CD results.

Toxicity values determined for the myotoxins fall around the range reported for E toxin (C. h. horridus) in the presence of acetate (Allen et al., 35). It may be argued that because of chromatography using acetate buffers, the basic amino acid side chains in these myotoxins have acetate bound as a counter-ion; hence their toxicity. Since the eluting cation exchange buffer contained only 20 mM acetate and 2 M NaCl, it is likely that Cl^-, rather than acetate, constituted the bulk of the counter-ion. Ownby (unpublished observations) has reported similar values for myotoxin a where no acetate was used in any purification step. We find the high LD_{50} value (low toxicity) reported (35), for E toxin in the absence of exogenous acetate (6.3 µg/g) difficult to explain. It may possibly be attributed to the one-step purification procedure they employed. Trace contamination with proteases might account for gradual loss of toxicity experienced previously with this toxin (Sullivan and Geren, 36).

Several noteworthy observations were made during toxicity assays. Using a dose of 4 µg/g, which is close to the LD_{50} reported for crotamine by Cheymol et al. (37), mice exhibited flaccid paralysis before the injection could be completed. Death ensued in less than 30 sec. Lethal doses closer to the LD_{50} caused death within 2 min.; however, if a mouse survived as much as 5 min., recovery seemed ensured. This is in strong contrast to crotalid presynaptic neurotoxins, which continue to kill test animals as much as 18-24 hr post-injection, despite the fact that their toxicity exceeds that of the myotoxins by more than an order of magnitude. Our observations on mice that received a lethal dose suggest that death may result from respiratory failure, perhaps caused by paralysis of the diaphragm and skeletal musculature controlling movement of the rib cage. Such a conclusion would appear to be supported by the effects of myotoxin a on the resting membrane potential of mouse and rat diaphragms (38). Myotoxins from C. v. concolor do not appear to induce death by cardiac failure. When sublethal doses of myotoxin a from C. v. viridis were injected into mice i.v., paraffin sections of cardiac muscle examined by light microscopy failed to reveal any evidence of myonecrosis (Ownby, unpublished observations). We noted that in mice that received a lethal dose, the heart beat strongly throughout and continued for several minutes after the cessation of all attempts to breathe.

CONCLUSIONS

1. The four monoclonal antibodies raised against the basic subunit of crotoxin have been further characterized for their cross-reactivity against other proteins (7-9). One is a potent neutralizer of crotoxin's lethality and phospholipase activity. It has been used to generate anti-idiotype monoclonal antibodies by Dr. John Middlebrook. None of the anti-idiotype antibodies examined so far have had detectable phospholipase activity.

2. A crotoxin homolog is present in the venom of C. vegrandis (10), but none was found in venom from either C. v. lutosis (11), or C. atrox/C. atrox-C. s. scutulatus (type B venom) hybrids (12).

3. Determination of the primary sequence of the basic subunit of crotoxin has been completed (14, 15).

4. A partial sequence for the acidic subunit of crotoxin was reported by us earlier (2). In collaboration with Dr. Don Hunt, we recently determined the remainder of this sequence (in preparation).

5. Spectral studies suggest that much, if not most, of the intramolecular subunit conformational changes occur in the basic phospholipase subunit. This would follow from the relatively high ß-sheet content of this subunit which would permit the significant loss of ß structure to readily occur. There is sufficient ß structure in the acidic subunits, however, that a possible role for them in ß structure reduction cannot be eliminated. Several pieces of evidence suggest that the striking conformational alterations observed upon complex formation play a role in the biological activity of the toxins. For example, when phospholipase kinetics of intact proteins are compared to those of free basic subunits, it is found that the intact toxins display sigmoidal behavior with a distinct lag phase while the latter manifest the expected hyperbolic curves (5). In addition, it is found from the crystal structure of the dimeric phospholipase A_2 of C. atrox venom that access to the substrate binding site is severely restricted by steric factors (34). Since this dimeric structure may be a good structural analogue to the crotalid heterodimeric complexes, it is possible that substrate accessibility could be modulated by the conformational changes in such a manner that kinetics are functionally controlled. Current work on the x-ray structures of crotoxin and Mojave toxin should clarify the role of these intriguing structure changes in the biological activity of these toxins (34, 19).

6. Muscle necrosis appears to be the most significant medical problem associated with the small myotoxins. Our observations on predatory behavior of C. v. concolor, C. v. viridis and C. v. helleri indicate that the biological significance of these molecules is their ability to halt the flight of a prey organism almost instantaneously. In many cases, a mouse struck by one of these snakes is unable to take a single step, and the hyperextension of the hind limbs that characterizes injections of purified myotoxins is readily observable.

7. We are attempting to apply bioluminescence methodology to acetylcholine detection, with possible future application to synaptosome systems. We have also collaborated with Dr. Lance Simpson on examining the affects of crotoxin and its neuromuscular blocking properties on the isolated phrenic nerve-hemidiaphragm.

8. A cDNA and genomic library have been prepared from C. d. terrificus in lambda in collaboration with Dr. Leonard Smith.

RECOMMENDATIONS

1. Continue our characterization of the monoclonal antibodies.
2. Continue our characterization of cross-linked crotoxin and determine its toxicity and phospholipase activity.
3. Continue our collaboration with Dr. Keith Ward on the determination of the crystal structure of Mojave toxin that is in progress. Determine the primary sequence of the basic subunit of Mojave toxin when the data are needed by Ward.
4. Continue attempts to establish an in vitro system using synaptosomes and bioluminescence for examining the biological effects of presynaptic neurotoxins on acetylcholine release. Maintain the collaboration with Dr. Lance Simpson, who is examining the affects of crotoxin on the phrenic nerve-hemidiaphragm.
5. Continue to characterize the cDNA and genomic clones of C. d. terrificus, as well as the genomic clones from C. atrox.

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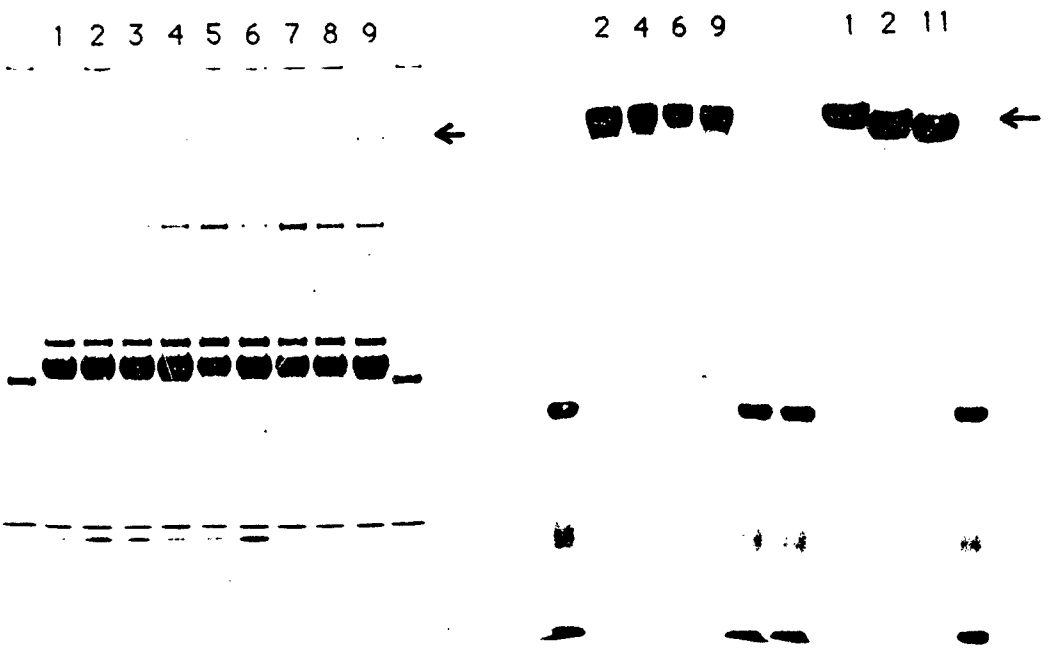

Fig. 1. Non-reducing SDS-PAGE (7.5% acrylamide) of anti-idiotype ascites fluid from mice inoculated i.p. with hybridoma cells generated against the purified, intact crotoxin neutralizing monoclonal antibody prepared by Kaiser and Middlebrook (8). About 20μg of protein was run per slot. The antibody region of the gel is indicated by the arrow. Assigned number and reference number are as follows: 1, 4D10-1G6-1D2 (5-12-87); 2, 7E11-2D4-2C8 (5-12-87); 3, 6G9-2E4-1D4 (5-12-87); 4, 10E6-1G2-1B5 (5-12-87); 5, 4F9-2B4-2D5 (5-20-87);6, 7B5-2E11-1B6 (5-12-87); 7, 10D8-1E7-1B8 (3-11-87); 8, 7E11-2D4-1B9 (1-9-87); 9, 7F9-1F4-1C2 (3-4-87).

Fig. 2. Non-reducing SDS-PAGE (7.5% acrylamide) of four purified, anti-idiotype monoclonal antibodies. Monoclonal antibodies from samples 2, 4, 6, and 9 were recovered from crude ascites fluid by protein-A chromatography. The antibody region of the gel is indicated by the arrow. Purified monoclonal antibodies 1,2, and 11, raised against the basic subunit of crotoxin are shown for comparison (ref. 8). About 20μg of protein was run per slot.

Table 1. Competition of neurotoxins, crude venoms, and other proteins with biotinylated-crotoxin for binding to monoclonal antibodies.

Materials tested	ID_{50} (ng/well) Monoclonal antibodies			
	Line 1	Line 2	Line 5	Line 11
Intact crotoxin	95	83	31	83
Basic subunit crotoxin (immunogen)	45	340	>1000	170
Acidic subunit crotoxin	1000	170	25	50
Mojave toxin	69	130	83	110
Concolor toxin	300	170	80	130
Vegrandis toxin	170	330	250	330
C. atrox phospholipase A_2	>1000	>1000	>1000	>1000
Taipoxin	>1000	>1000	>1000	>1000
C. d. terrificus venom	160	550	77	250
C. v. concolor venom	1000	720	290	480
Oxyuranus s. scutellatus venom	>1000	>1000	>1000	>1000
Bungarus multicinctus venom	>1000	>1000	>1000	>1000
Pseudonaja textillis venom	>1000	>1000	>1000	>1000
Notechis scutatus venom	>1000	>1000	>1000	>1000

Proteins were examined for their binding to the indicated plate-bound monoclonal antibody in the presence of biotinylated-crotoxin. ID_{50}-values correspond to the concentration of competing protein which inhibited 50% of biotinylated-crotoxin binding (STRONG et al., 39)

Table 2. Secondary structure of four crotalid presynaptic neurotoxins as estimated from circular dichroism data by the method of Provencher and Gloeckner (1981). Secondary structure of intact toxins was predicted using molecular weights of 9,213 and 14,200 from the acidic and basic subunits of crotoxin, respectively (Aird et al. 2, 14). Due to rounding error, percentages do not always total 100%.

Taxon - Protein	% α-helix	% β-sheet	% Remainder
C. d. terrificus- Acidic Subunit	37	29	35
C. d. terrificus - Basic Subunit	16	51	33
C. d. terrificus - Pred. Intact Toxin	24	42	34
C. d. terrificus- Obs. Intact Toxin	29	23	48
C. vegrandis - Acidic Subunit	22	40	38
C. vegrandis - Basic Subunit	20	40	40
C. vegrandis - Pred. Intact Toxin	21	40	39
C. vegrandis - Obs. Intact Toxin	26	36	38
C. s. scutulatus - Acidic Subunit	34	39	26
C. s. scutulatus - Basic Subunit	22	35	43
C. s. scutulatus - Pred. Intact Toxin	27	37	36
C. s. scutulatus - Obs. Intact Toxin	28	29	43
C. v. concolor - Acidic Subunit	49	27	25
C. v. concolor - Basic Subunit	16	49	35
C. v. concolor - Pred. Intact Toxin	29	40	31
C. v. concolor - Obs. Intact Toxin	28	32	39

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